

# Cloning and expression of a $\gamma$ -interferon-inducible gene in monocytes: a new member of a cytokine gene family

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**Key words.** subtractive hybridization, anti-CD2 antibodies, T cell activation, activation cascade

## Abstract

To define activation-specific sequences in human peripheral blood lymphocytes (PBL), a cDNA library was constructed by subtractive hybridization using resting and stimulated PBL pairs. Stimulation of PBL was achieved by triggering with mitogenic anti-CD2 (T11) monoclonal antibodies. Differential library screening with cDNA probes derived from stimulated versus resting PBL led to identification of two novel sequences, termed HC11 and HC14. The predicted primary and secondary structure of HC11 deduced from the translated nucleotide sequence suggests that the gene encodes a secreted protein of 99 amino acids (aa), including a 23 aa residue leader sequence. Surprisingly, Northern blot analysis demonstrated that HC11 mRNA is induced predominantly in peripheral blood non-T cells. Subsequently, we observed that the HC11 mRNA is induced in macrophages and the monocytic line U-937 by  $\gamma$ -IFN, raising the possibility that T cell-derived  $\gamma$ -IFN induced upon anti-CD2 stimulation activated monocytes to express HC11 RNA. In support of this notion, neutralizing anti- $\gamma$ -IFN monoclonal antibody inhibits the induction of HC11 mRNA in PBL activated through anti-CD2 antibodies. These findings suggest that there is a molecular cascade involving T cell-produced lymphokines and monokines which serve as a means for intercellular communication. Transient expression of HC11 cDNA results in a readily detectable specific set of protein bands in SDS-PAGE analysis of supernatants from radio-labeled COS cells, consistent with HC11 encoding a secreted product(s). Protein sequence comparison reveals homology with other members of a recently described inducible cytokine family whose functions are yet to be defined.

## Introduction

The activation of T lymphocytes involves the orderly induction of gene programs effecting cell growth, regulatory, and effector functions (reviewed in 1). To begin to define these activation-specific sequences in human T lymphocytes, we and others have utilized cDNA libraries constructed by subtractive hybridization techniques and differential library screening with cDNA probes derived from stimulated and resting lymphocytes or clones (2–10).

In the present study we have employed this technology to identify two additional structural genes which encode secreted proteins, presumed to be cytokines. Interestingly, these putative cytokines are induced in monocytes by gamma interferon ( $\gamma$ -IFN) stimulation resulting from CD2-mediated T cell activation. One of the cytokines, termed HC11, has been characterized at both

the cDNA and genomic levels, and its protein expressed in COS cells. Protein sequence comparison between HC11 and other sequences reveals substantial homology of HC11 with members of a cytokine family which have remarkably conserved structural features.

## Methods

### Cells

Human peripheral blood lymphocytes (PBL;  $2-4 \times 10^9$  cells) were isolated by the Ficoll-Hypaque method from buffy coat preparations and T lymphocytes (E<sup>+</sup>) were separated from B and macrophage (E<sup>-</sup>) cells by rosetting with sheep erythrocytes. Macrophages were separated by repeated

adherence steps of the E<sup>-</sup> fraction as described (11). Purity was determined by immunofluorescence analysis with monoclonal antibody (mAb) to the Mo1 (CD16) or Mo2 (CD14), T3 (CD3) or T11 (CD2), B1 (CD20), and NKH1-A antigen (12). The purity of T lymphocytes was usually <95% and that of macrophages usually >90%. The monocytic cell line U-937 or the monomyelocytic cell line HL-60 and human T cell tumor line REX were maintained as described (10).

#### Activation conditions

To stimulate T lymphocytes, cells were suspended in final medium [RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% human AB serum (PelFreeze, Rogers, AR), 2% glutamine (200 mM) and 1% penicillin-streptomycin (Whittaker MA Bioproducts, Walkersville, MD)] at  $1 \times 10^6$  cells/ml and cultured with either medium or anti-T11<sub>2</sub> + anti-T11<sub>3</sub> monoclonal antibodies at a final dilution of 1:100 ascites for various times. To activate monocytes, U-937, or HL-60 cells, the cells were cultured at  $1 \times 10^6$  cells/ml in 60 × 15 mm Petri dishes at 37°C for 24 h in the presence or absence of 200–2000 U/ml (sp. act. =  $2 \times 10^7$  U/mg) purified *Escherichia coli*-derived  $\gamma$ -IFN (Biogen) or 12-O-tetradecanoylphorbol-13-acetate (TPA) at  $5 \times 10^{-8}$  or  $1 \times 10^{-7}$  M or dimethylsulfoxide (DMSO) at 1.5%.

#### Production of an activation specific cDNA library

To construct a cDNA library enriched in activation sequences, a subtractive hybridization between cDNA from activated PBL and poly(A)<sup>+</sup> RNA from resting PBL was used as described in detail previously (10). In brief, PBL were cultured at  $1 \times 10^6$  cells/ml in final medium with anti-T11 antibodies for 36 h, after which cytoplasmic RNA was extracted by the NP-40 lysis method (13) and poly(A)<sup>+</sup> RNA isolated by oligo(dT) chromatography. Single-stranded cDNA (ss-cDNA) was synthesized and freed from template RNA by the alkaline method as described (14). To enrich for activation sequences, 1  $\mu$ g of ss-cDNA was hybridized with 70  $\mu$ g poly(A)<sup>+</sup> RNA from resting PBL at  $C_{ot} > 4000$  mol s/l in 0.41 M sodium phosphate buffer, pH 6.8, containing 0.1% NaDodSO<sub>4</sub> and 1 mM EDTA at 68°C for 20 h. The samples were then applied to an hydroxyapatite column (Biorad, Richmond, VA) at 60°C and the unbound ss-cDNA was eluted with 0.12 M sodium phosphate, pH 6.8. Approximately 100 ng of unbound ss-cDNA was recovered (~10-fold enrichment without taking into account non-specific loss). The second strand of cDNA was synthesized, C-tailed, annealed with G-tailed pBR322 as described (15), and used to transform competent *E. coli* HB101 (BRL), resulting in a subtracted cDNA library consisting of ~5000 colonies.

#### Production and differential screening with cDNA probes

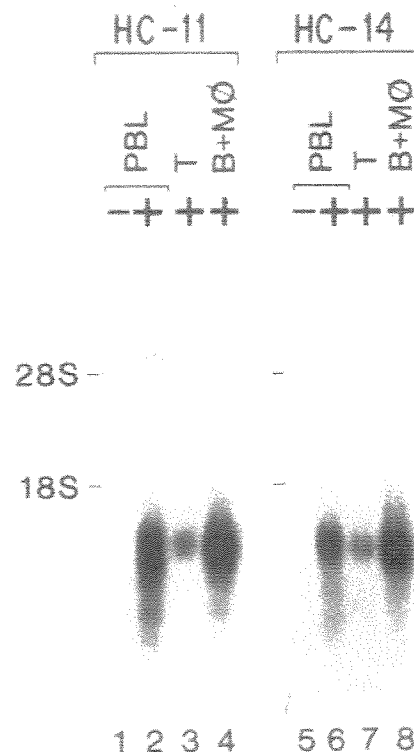
To isolate cDNAs encoding sequences specific for activation, differential screening with radioactive probes was performed as described previously (10). The <sup>32</sup>P-labeled cDNA was synthesized from the poly(A)<sup>+</sup> RNA of anti-T11 activated PBL. The ss-cDNAs were hybridized and selected twice through hydroxyapatite columns with poly(A)<sup>+</sup> RNA from resting PBL at  $C_{ot} > 2000$ . The unbound ss-cDNAs were used as probes for activated sequences at a sp. act. of  $1 \times 10^8$  cpm/ $\mu$ g. The probe for resting or non-activated sequences represents the cDNAs synthesized from poly(A)<sup>+</sup> RNA of resting PBL at a sp. act. of

$1 \times 10^8$  cpm/ $\mu$ g. Both activated and non-activated probes were used in parallel to screen duplicates of seven 137 mm nitrocellulose filters (Millipore, Bedford, MA), each containing ~800 colonies of the lymphocyte activation specific cDNA library.

#### A full-length cDNA and genomic library

To isolate the entire coding sequence, cytoplasmic poly(A)<sup>+</sup> RNA from activated PBL was used to construct a cDNA library according to Gubler and Hoffman (16). The double-stranded cDNA was ligated to *Eco*RI linker, then cloned into p48 vector (provided by Dr T. Roberts, Dana-Farber Cancer Institute, Boston, MA), and used to transform competent *E. coli* HB101 (BRL). The library was then screened with both fragments of the HC11 probe to generate clone HC11-6. A second full-length cDNA library, derived from PHA-activated PBL in the pcD vector, was screened with HC11-1 and HC11-6 probes, and produced HC11-26 and HC11-30 clones.

About 10,000 colonies of a human leukocyte genomic library in the cosmid vector pCV105 and propagated in *E. coli* host DK (kindly provided by Dr Y. F. Lau, Howard Hughes Medical Institute, University of California, San Francisco, CA; 17) were



**Fig. 1.** Analysis of HC11 and HC14 RNA expression. Human PBL were cultured with or without anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies for 24 h. The cells were then separated by rosetting with SRBC into E rosetting-positive populations (T cells) and E rosetting-negative populations (B cells + MØ). 20  $\mu$ g cytoplasmic RNA from non-activated PBL (lanes 1, 5), activated PBL (lanes 2 and 6), activated T lymphocytes (lanes 3, 7), and activated B cells + macrophages (lanes 4 and 8) were used in Northern analysis and probed with <sup>32</sup>P-labeled HC11-1 insert (lanes 1–4) and <sup>32</sup>P-labeled HC14 (lanes 5–8). Positions of 28S and 18S rRNA are indicated. Autoradiograms were exposed 16 h at –70°C with an intensifying screen.

plated out and screened with both *Pst*I fragments of the HC11-1 probe. Three positive cosmid clones were digested with *Bam*HI, then shotgun subcloned into the pUC18 vector (IBI, New Haven, CT) and screened for HC11 sequence containing clones which were used for further DNA sequence analysis.

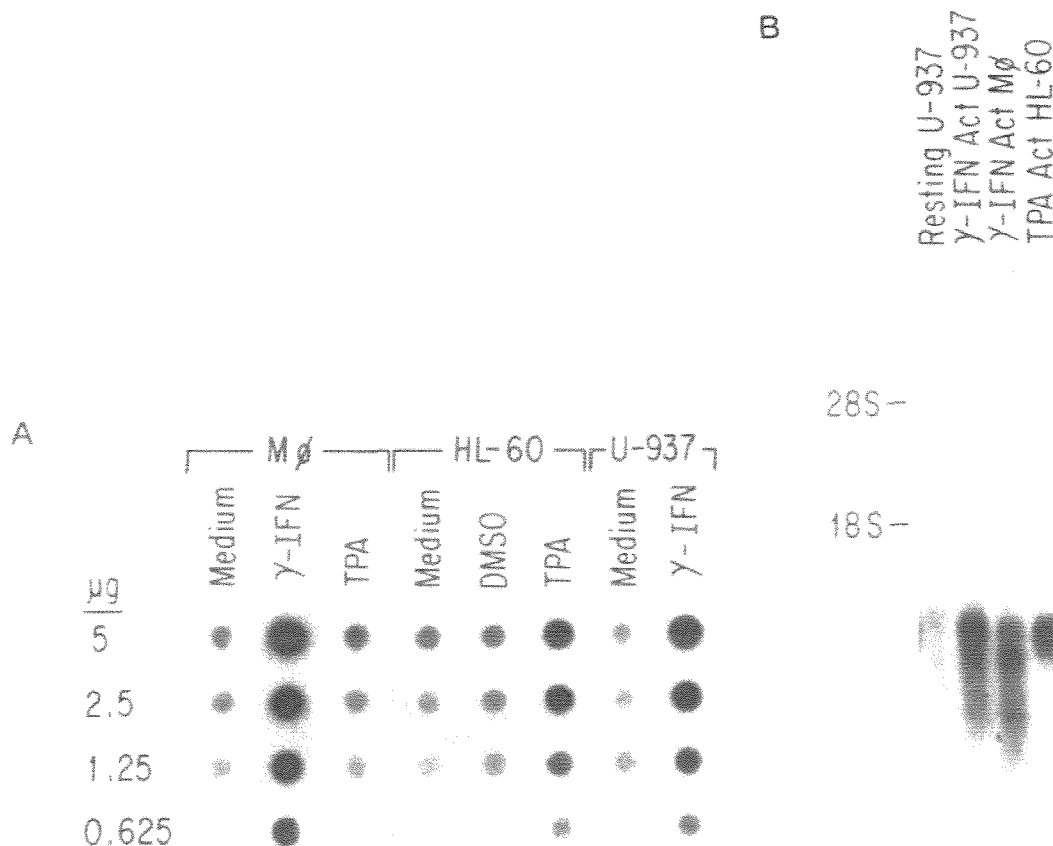
#### Northern and Southern blot analysis

Cytoplasmic RNAs were isolated from a variety of cells after NP-40 lysis. The RNA was either dot blotted onto Gene Screen Plus filter (NEN, Boston, MA) or size fractionated on 1.3% formaldehyde denaturing agarose gels in MOPS buffer (13) and transferred to a Gene Screen Plus filter. The cellular DNAs of the T cell tumor REX (a non-IL-2 producing Jurkat variant) was isolated and 10  $\mu$ g of each DNA were digested with *Bam*HI, *Eco*RI, or *Hind*III and run on 0.8% agarose gel in Tris-acetate buffer at 25 V for 20 h. DNA digests were then denatured, neutralized, and transferred onto Gene Screen Plus filters as described (13). Both RNA and DNA blots were hybridized in plaque screen buffer containing 50 mM Tris, pH 7.5, 1 M NaCl, 1% NaDodSO<sub>4</sub>, 0.1% sodium pyrophosphate, 0.2% bovine serum albumin, 0.2% Ficoll, and 0.2% polyvinylpyrrolidone at

68°C for 20 h with the <sup>32</sup>P-labeled probe ( $1 - 5 \times 10^6$  cpm/ml). To generate the probes, plasmid preparations of HC11 and HC14 clones were digested with *Pst*I and size-fractionated on low melting point agarose gel (BRL). Insert bands were cut out and labeled directly in the gel slice by the random priming method (18) to a sp. act.  $> 1 \times 10^8$  cpm/ $\mu$ g. Autoradiography was done with Kodak XAR 5 film and intensifying screens (Eastman Kodak, Rochester, NY) for various times. Densitometry scanning of Northern blots was performed by Quick Scan R&D (Helena Lab, Beaumont, TX) and expressed as % of maximal intensity.

#### DNA and protein sequence analysis

For HC11 cDNA clones, HC11-1, HC11-6, HC11-26, and HC11-30, were subcloned into M13mp18 and mp19 and sequenced by the [<sup>35</sup>S]ATP-labeled dideoxy method (19) using either M13 primer or synthetic oligonucleotide primers corresponding to cDNA sequences 7-24, 163-180, 629-646, and 728-740 bp. The latter were made on an ABI 381A DNA synthesizer (Applied Biosystems, Foster City, CA) with cyanoethylphosphoramidite chemistry. The longest DNA sequence of HC11-30 was compared against published



**Fig. 2.** Induction of the HC11 gene. (A)  $20 \times 10^6$  cells of human peripheral macrophages were cultured at  $1 \times 10^6$  cells/ml in the presence or absence of 500 U/ml of  $\gamma$ -IFN or  $10^{-7}$  M TPA for 24 h.  $20 \times 10^6$  HL-60 cells were cultured with 1.5% DMSO, or  $5 \times 10^{-8}$  M of TPA.  $20 \times 10^6$  U-937 cells were cultured with 500 U/ml of  $\gamma$ -IFN. Cytoplasmic RNA was obtained from each stimulated cell population and 5, 2.5, 1.25, and 0.625  $\mu$ g in  $15 \times$  SSC applied onto a Gene Screen Plus filter for dot blot analysis. (B) 20  $\mu$ g of cytoplasmic RNA from the resting U-937,  $\gamma$ -IFN-activated U-937 cells,  $\gamma$ -IFN-activated macrophages, and TPA-activated HL-60 cells as indicated above were subjected to size electrophoresis and hybridized with the same HC11 probe. The position of 28S and 18S rRNA are indicated.

sequences compiled in the GenBank release 57 NIH using the Dasher program [D. V. Faulkner and T. F. Smith, Molecular Biology Computer Research Resource, DFCI, Harvard, 1987. This program uses a modified Wilbur-Lipman algorithm (40)]. The HC11 and HC14 protein sequences were compared with known proteins by searching against translated gene bank (GB 57 seq) and protein bank (nbrf-18 seq 14028 sequences) using the Dasher program. Secondary structural predictions were analysed in the PC gene protein analysis program by the Chou-Fasman rules.

The DNA sequence analysis of genomic clones were carried out by subcloning the cosmid clones of HC11 into the *Bam*HI site of pUC18. The pUC18 *Bam*HI clone, which contains 9.4 kb of *Bam*HI insert, was digested with *Hinc*II and subcloned into the *Hinc*II site of pUC18. One of the subclones, termed pUC 7 kb, containing an ~7 kb fragment, was subjected to DNA sequence

analysis by either single-strand sequencing using M13 vector or direct double-strand sequencing. The primers used in the sequencing were either the universal primers of M13 vector or synthetic oligonucleotide primers which were derived from the cDNA or genomic sequence.

#### COS cell expression

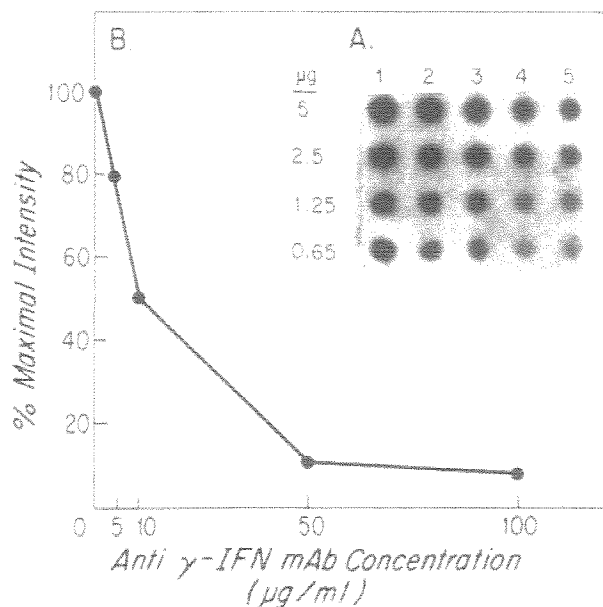
The entire coding region of HC11 (the 5' end of the *Pst*I fragment) was subcloned into the pmt2 vector in both orientations. This vector is a derivative of pXM (20) and was a gift of G. G. Wong (Genetics Institute, Cambridge, MA) and B. Rollins (Dana-Farber Cancer Institute, Boston, MA). Aliquots (10 µg each) of pmt2-HC11 in the sense orientation and anti-sense orientation were transfected into COS-1 cells at 60% confluence by DEAE-dextran method, followed by chloroquine treatment as described (7). After 48 h, medium was replaced by cysteine-free RPMI medium containing 500 µCi of [<sup>35</sup>S]cysteine (800 Ci/mmol; 1 Ci = 37 BGq). Medium was collected and cells were spun out on a microcentrifuge. A 30 µl quantity of medium from each transfection was then used for protein analysis in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The gel was fixed, stained, destained, enhanced, and dried as described (21). It was autoradiographed for 24 h.

## Results and discussion

#### Isolation of activation-related cDNAs

To isolate cDNA clones representing gene sequences induced during lymphocyte activation, we constructed a cDNA library enriched in activation sequences as described in the Methods. This was differentially screened with a subtracted 'activation' probe derived from anti-T11<sub>2</sub> + anti-T11<sub>3</sub>-stimulated PBL and a 'non-activated' probe derived from resting PBL of the same donor. The clones which hybridized with a subtracted probe but were unreactive with the non-activated probe were isolated and further analysed. From 5000 colonies screened, 16 demonstrated a differential pattern of hybridization after rescanning with a second set of activation and resting probes. Plasmid preparations of each of these 16 clones were produced, DNA digested with *Pst*I and inserts isolated by agarose gel electrophoresis. <sup>32</sup>P-labeled inserts from individual clones were used as probes in Northern analysis. Inserts from several of these clones hybridized with activated PBL RNA but not resting PBL. Southern analysis of these clones was carried out to detect inserts which cross-hybridize. Two groups of clones were identified and representatives of each of these two groups containing the longest inserts, termed HC11 and HC14, were subjected to further analysis.

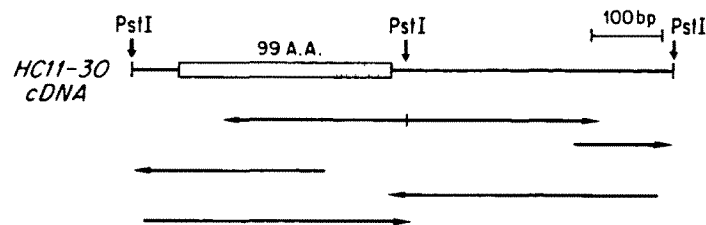
The HC11 and HC14 inserts hybridized to RNA species of ~800 bp in size expressed in PBL which had been activated



**Fig. 3.** Inhibitory effect of anti-γ-IFN monoclonal antibody on induction of HC11 message.  $20 \times 10^6$  human PBL were cultured with anti-T11<sub>2</sub> + anti-T11<sub>3</sub> antibodies in the presence or absence of various amounts of anti-γ-IFN monoclonal antibody (3C11C8) (25) for 25 h. (A) Cytoplasmic RNA was isolated and dot blot analysis performed as in Fig. 2. The RNAs of PBL activated by anti-CD2 mAbs with no anti-γ-IFN antibody (lane 1) or in the presence of 5 (lane 2), 10 (lane 3), 50 (lane 4), and 100 µg/ml (lane 5) of anti-γ-IFN mAb were hybridized with HC11 probe. The amount of RNA spotted at each point on the filter is indicated on the left. Autoradiograms were exposed for 16 h at -70°C with an intensifier screen. (B) Densitometry scanning of the autoradiogram in (A) in terms of % HC11 RNA signal induced in activated PBL without anti-γ-IFN monoclonal antibody.

**Fig. 4.** DNA sequencing strategy and protein structure. (A) Sequencing strategy for HC11. The HC clone 11-30 was digested with *Pst*I to generate two *Pst*I fragments, subcloned into *Pst*I site of M13mp18-19, and sequenced in both strands by using M13 universal primer or synthetic oligonucleotide derived from cDNA sequence as described in Methods. (B) Nucleotide sequence and translated protein sequence of HC11-30. The predicted amino acid sequence of HC11-30 is shown beneath the DNA sequence from nucleotide 70 to 366 bp in three letter code. The putative cleavage site of leader peptide is indicated by an arrow. The polyadenylation signal is underlined. The potential N-glycosylation site is boxed. The exon-intron boundaries are indicated by a vertical line. The numbering of amino acids (right of figure) is based on the predicted mature protein sequence. (C) Hydropathicity plots of HC11. The y axis represents hydropathicity values derived by Chou-Fasman rule. The x axis shows position along the amino acid sequence.

A



B

10 20 30 40 50 60  
CCCCCCCAGG CTGAGACTAA CCCAGAAACA TCCAATTCTC AAAGTGAAGC TCGCACTCTC GCCTCCAGC

84 99 114  
ATG AAA GTC TCT GCC GCC CTT CTG TGC CTG CTC ATA GCA GCC ACC TTC ATT  
MET Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Ile Ala Ala Thr Phe Ile -6

129 144 159 174  
CCC CAA GGG CTC GCT CAG CCA GAT GCA ATC AAT GCC CCA GTC ACC TGC TGT TAT  
Pro Gln Gly Leu Ala Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr 13

189 204 219  
AAC TTC ACC AAT AGG AAG ATC TCA GTG CAG AGG CTC GCG AGC TAT AGA AGA ATC  
[Asn] Phe Thr Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile 31

234 249 264 279  
ACC AGC AGC AAG TGT CCC AAA GAA GCT GTG ATC TTC AAG ACC ATT GTG GCC AAG  
Thr Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala Lys 49

294 309 324 339  
GAG ATC TGT GCT GAC CCC AAG CAG AAG TGG GTT CAG GAT TCC ATG GAC CAC CTG  
Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser MET Asp His Leu 67

354 369 379 389 399  
GAC AAG CAA ACC CAA ACT CCG AAG ACT TGA ACACTCACTC CACAACCCAA GAATCTGCAG  
Asp Lys Gln Thr Gln Thr Pro Lys Thr . 76

409 419 429 439 449 459 469  
CTAACTTATT TTCCCCTAGC TTTCCCAGCA CACCCTGTTT TATTTTATTA TAATGAATTT TGTTTGTGA

479 489 499 509 519 529 539  
TGTGAAACAT TATGCCTTAA GTAATGTTAA TTCTTATTTA AGTTATTGAT GTTTTAAGTT TATCTTTCAT

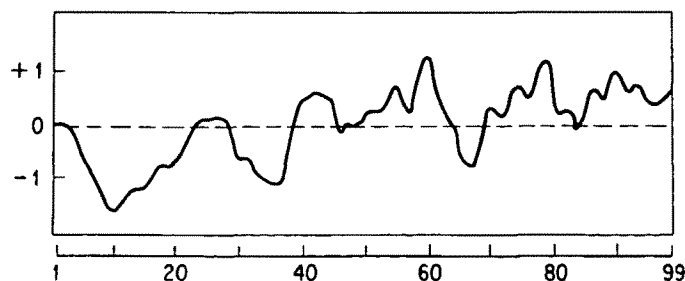
549 559 569 579 589 599 609  
GGTACTAGTG TTTTITAGAT ACAGAGACTT GGGGAAATTG CTTTTCCTCT TGAACCACAG TTCTACCCCT

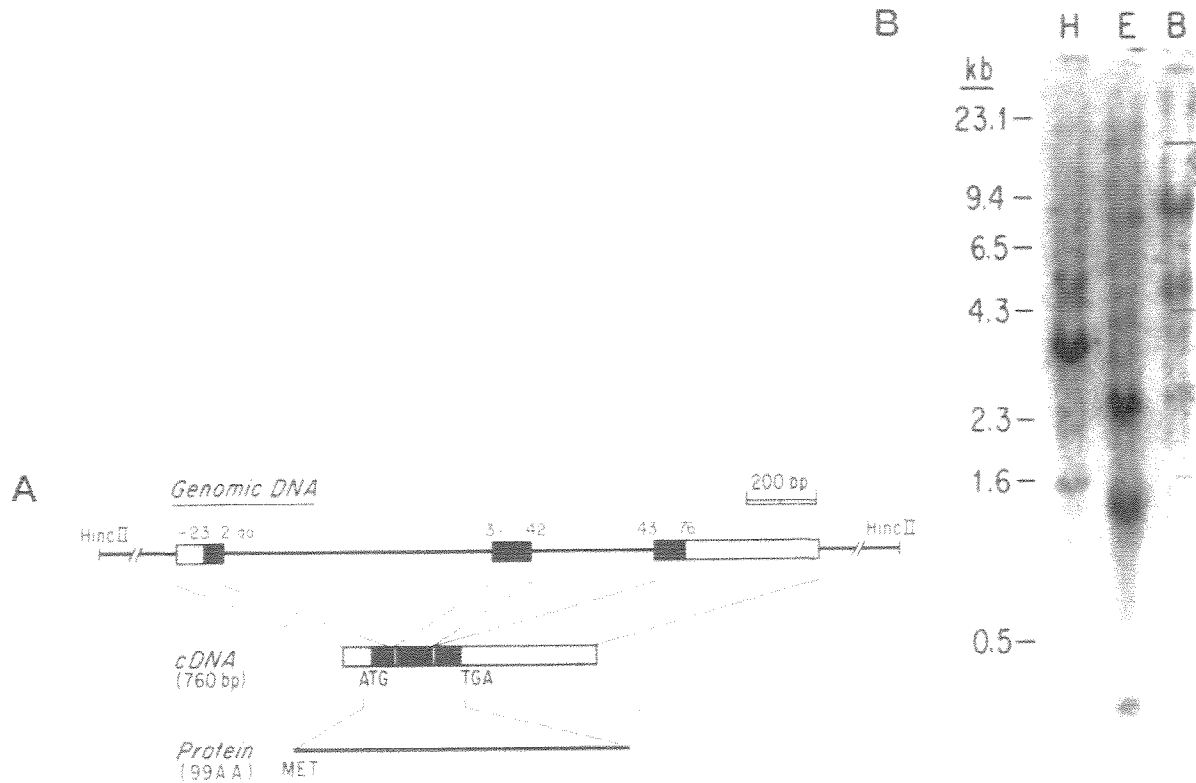
619 629 639 649 659 669 679  
GGGATGTTTT GAGGGTCTTT GCAAGAATCA TTAATACAAA GAATTTTTTT TAACATTCCA ATGCATTGCT

689 699 709 719 729 739 749  
AAAATATTAT TGTGGAAATG AATATTTTGT AACTATTACA CCAAATAAAT ATATTTTGTG AAAAAAAAAA

759  
AAAAAAAAA AAA

C

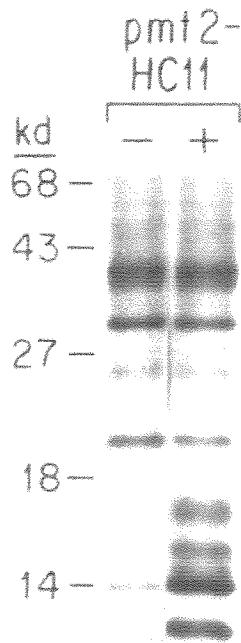




**Fig. 5.** Genomic organization and Southern analysis of HC11 gene. (A) The intron-exon organization of human HC11 gene. The shaded and stippled areas denote the coding and untranslated regions of the HC11 gene, respectively. The amino acids were numbered relative to the predicted mature protein. Since the RNA start site has not been mapped, the 5' end of exon 1 is unknown. Exon 1 ends at nucleotide 145 (cDNA numbering). Exon 2 is 118 bp in size (nucleotides 146–263) and exon 3 is 471 bp in size. Introns 1 and 2 are 798 and 381 bp, respectively. Donor and acceptor sequences for exons are as follows: GTAAGGCCCC---TATTTCCAG in the first intron and GTGAGTTCAG---CTCCCCACAG in the second intron. (B) Southern analysis of HC11 gene in the human T cell tumor line REX (a Jurkat subclone). DNA samples were extracted from the human T cell line REX. 10  $\mu$ g of each DNA was digested with *Bam*HI (B), *Eco*RI (E), or *Hind*III (H) and run for 20 h on a 0.8% agarose gel at 20 V. The Southern blot was hybridized with both  $^{32}$ P-labeled *Pst*I fragments of the HC11-1 insert. Size markers were derived from lambda *Hind*III digests (NE Biolabs, Beverly, MA). The autoradiogram was exposed for 48 h at  $-70^{\circ}\text{C}$  with intensifier screen.

for 36 h previously by anti-T11<sub>2</sub> and anti-T11<sub>3</sub> antibodies (Fig. 1, lanes 2 and 6). In contrast, no hybridization was observed with RNA isolated from resting PBL (Fig. 1, lanes 1 and 5). To examine which population of activated cells contains these sequences, the PBL were activated for 36 h with anti-CD2 mAbs, then separated into T lymphocyte (E rosette positive) and B plus macrophage cellular fractions (E rosette negative) and subsequently the RNAs were isolated and subjected to Northern analysis. The results shown in Fig. 1 demonstrated that both HC11 (lane 4) and HC14 (lane 8) were expressed predominantly in the population containing B cells plus macrophages. In contrast, little specific HC11 or HC14 RNA was detected in purified T lymphocytes (lanes 3 and 7 respectively). It was surprising that HC11 and HC14 sequences were induced in non-T cells in view of the fact that the activation of PBL was stimulated via the CD2 molecule in T lymphocytes. To resolve this paradox and study the mechanism of their induction, we individually separated the B cell and macrophage populations first and then examined the effects of different stimuli on HC11 and HC14 gene induction.

To study gene expression in macrophages, the macrophages were isolated from PBL as described in the Methods and activated with 500 U/ml of  $\gamma$ -IFN or  $1 \times 10^{-7}$  M of TPA for 24 h. Subsequently, the RNA was isolated and spotted onto a Gene Screen plus filter and hybridized with  $^{32}$ P-labeled HC11 probe. The resulting autoradiograph of the dot blots shown in Fig. 2(A) demonstrated that the HC11 gene can be induced in macrophages by  $\gamma$ -IFN but not by TPA. A similar pattern was observed with HC14 (data not shown). Consistent with the finding on PBL-derived monocytes, the results shown in Fig. 2(A) demonstrate that HC11 can be induced in the monocytic line U-937 by  $\gamma$ -IFN as well as in the monomyelocytic HL-60 cells by TPA, but not by DMSO. Of note, it has been demonstrated that HL-60 can be induced to selectively differentiate into macrophage by TPA whereas DMSO drives HL-60 to differentiate along a granulocytic pathway (22). This result suggests that the induction of the HC11 gene by  $\gamma$ -IFN is monocyte lineage-related. To characterize the size of the RNA species hybridizing with the HC11 insert, the RNA samples from macrophages activated with  $\gamma$ -IFN, U-937 cells activated with  $\gamma$ -IFN, and HL-60 cells activated



**Fig. 6.** Expression of HC11 protein in COS cells. The 5' end *Pst*I fragment of HC11-30 containing the entire coding region was subcloned into *Pst*I site of pmt2 vector in both orientations and transfected into COS-1 cells. 30  $\mu$ l of supernatant from [ $^{35}$ S]cysteine-labeled transfectants were electrophoresed on a 15% polyacrylamide gel in NaDodSO<sub>4</sub> under reducing conditions. The orientation of transfected DNA was indicated as '+' for sense orientation and '-' for anti-sense orientation. Positions of molecular weight markers are given on the left. (68 kd = bovine serum albumin; 43 kd = ovalbumin; 27 kd = carbonic anhydrase; 18 kd =  $\beta$ -lactoglobulin; and 14 kd = lysozyme).

with TPA were subject to electrophoresis and Northern analysis. The results in Fig. 2(B) show that 800 bp HC11-related RNAs are induced in all three cell lines by either  $\gamma$ -IFN or TPA. Of interest, there is substantial heterogeneity in size of hybridizing mRNA species in  $\gamma$ -IFN-activated U-937 cells and macrophages. Whether these results represent alternative forms of HC11 message or related but distinct RNAs encoded by separate genes is presently unknown. Although not shown, peripheral B cells, either resting or activated 24 h previously by Protein A-Sepharose, did not express HC11.

#### Identification of an immune response activation cascade

Given that CD2 is T lineage restricted (23) and the HC11 mRNA was derived from activated monocytes following stimulation of PBL with anti-CD2 mAbs, we suspected that a soluble mediator was responsible for induction of the monocyte gene. One potential lymphokine candidate based upon the above results was  $\gamma$ -IFN. In this respect, it is known that CD2 stimulation results in production of lymphokines, including  $\gamma$ -IFN and IL-2 (24). To test this possibility, we examined the effects of a neutralizing anti- $\gamma$ -IFN monoclonal antibody on PBL cultures when added prior to stimulation by anti-T11<sub>2</sub> + anti-T11<sub>3</sub> mAbs. After 24 h of

culture with anti- $\gamma$ -IFN mAb, RNAs were isolated and induction of HC11 gene examined by dot blot. As shown by the dot blot analysis and quantitative densitometry scanning in Fig. 3, the level of steady-state HC11 mRNA varied inversely with the concentration of anti- $\gamma$ -IFN antibody added. For example, where 5  $\mu$ g/ml of anti- $\gamma$ -IFN antibody was added into the culture of PBL prior to activation, only 80% of HC11 RNA expression obtained in the absence of anti- $\gamma$ -IFN antibodies was found (Fig. 3B). When increasing amounts of anti- $\gamma$ -IFN antibody were added into the culture, HC11 expression decreased such that at 100  $\mu$ g/ml anti- $\gamma$ -IFN mAb, only 7% of HC11 expression was obtained. Note that an irrelevant mAb, anti-T6, failed to affect HC11 gene induction in this system, even at 100  $\mu$ g/ml concentration (data not shown). We conclude that CD2 stimulation of PBL resulted in  $\gamma$ -IFN production by T lymphocytes followed by activation of macrophages via  $\gamma$ -IFN and, subsequently, HC11 induction in the macrophages.

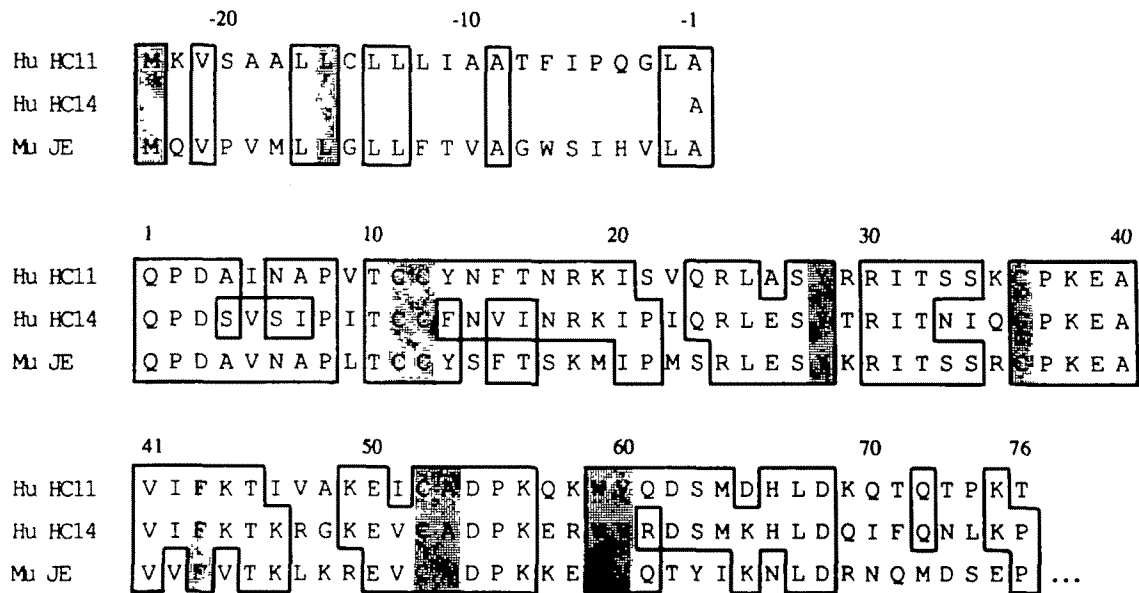
#### Nucleotide and protein sequence analysis of HC11

To obtain primary structural information on the HC11 gene, a cDNA library was prepared from total cytoplasmic poly(A)<sup>+</sup> RNA of activated PBL by the method of Gubler and Hoffman (16) in the p48 vector. This library was screened with the 300 bp 5' end *Pst*I fragment of HC11-1 cDNA insert as a probe. The hybridizing colonies were subcloned into the *Pst*I site of M13mp18-19 and subjected to DNA sequence analysis as described in the Methods and Fig. 4(A). The nucleotide sequence and predicted protein sequence of the longest clone, termed HC11-30, are shown in Fig. 4(B). The HC11 sequence contains an open reading frame of 297 bp (position 70–366) starting at the ATG codon at position 70–72 bp and terminating at the TGA at position 367–369 bp, followed by 393 bp of 3' untranslated region. One polyadenylation signal (AATAAA) is present at position 723–728 followed by a stretch of 21 A bases beginning at position 742. The putative amino acid sequence encodes a protein of 99 amino acids with a predicted molecular weight of 11,025 daltons. The 3' untranslated region is A and T rich and contains three copies of TTATT sequence at positions 439–443, 503–507, and 512–516, which is related to the 3' untranslated consensus sequence 'TTATTAT' defined by Caput *et al.* (26) as being a characteristic of many immunomodulatory proteins with a short RNA half-life.

Secondary structure analysis of predicted HC11 protein is shown in Fig. 4(C). A hydropathicity plot of HC11 protein, based on Chou–Fasman rules (27), shows that the predicted protein contains an N-terminal hydrophobic sequence (residues 1–23) consistent with a eukaryotic signal sequence (predicted by a von Heijne score of 10.22; 28). The predicted mature protein begins at aa 24 and is hydrophilic with no apparent transmembrane domain. This analysis suggests that HC11 gene encodes a secretory protein whose mature product is 76 aa in length and contains four cysteine residues at positions 11, 12, 36, and 52. There is one potential N-linked glycosylation site at position 14 (Asn-Phe-Thr). The majority of charged amino acids are located in the C-terminal region of the molecule.

#### The genomic structure and Southern blot analysis of HC11 gene

Genomic clones of HC11 were isolated from a cosmid library of human T lymphocyte DNA in the pCV105 vector by using both



**Fig. 7.** Comparison among HC11, HC14, and JE protein sequences. The predicted protein sequence of HC11 (this work), HC14 (this work and our unpublished data), and JE (7) were aligned. Amino acids that are identical between two or more sequences are boxed. The amino acids conserved among all members of inducible cytokine family are shaded. Prefixes refer to species: Hu, human; Mu, murine. The HC14 cDNA sequence is available upon request.

**Table 1.** Inducible cytokine gene/inflammatory protein family

Gene/protein	Source/activation stimuli	Ref
HC11 and HC14	human peripheral macrophage induced by $\gamma$ -IFN	This work
HC21	human peripheral T lymphocytes induced by anti-T11 <sub>2</sub> and anti-T11 <sub>3</sub> antibodies	10
LD78	human tonsillar lymphocytes induced by TPA or T cell mitogen, PHA	2
Rantes	human cytotoxic clone AH2, induced by antigen or mitogen	4
MIP-1 $\alpha$ , MIP-1 $\beta$ (macrophage inflammatory protein)	murine macrophage tumor line RAW264 induced by LPS	5,6
TCA-3	murine T <sub>H</sub> 1 T cell clone induced by Con-A	3
JE	murine fibroblast 3T3 cells induced by platelet-derived growth factor (PDGF)	7

**Table 2.** The homology between HC11 nucleotide and predicted protein sequence with other members of a cytokine family

HC11-30	Protein (identity ratio, %)	Nucleotide sequence similarity score	
		Coding region	Entire cDNA sequence
HC14	62.3	128.24	191.52
HC21	34.3	65.89	74.82
Rantes	31.3	40.82	44.31
LD78	35.3	56.40	65.25
MIP 1 $\alpha$	36.3	55.36	71.55
MIP 1 $\beta$	34.3	62.31	70.80
TCA-3	27.2	33.31	41.41
JE	51.5	127.75	176.56

The similarity scores are derived from comparison of the nucleotide sequences of the coding region or entire cDNA sequence of HC11-30 with the sequence of the indicated gene by using the LOCAL sequence comparison algorithm (30). The similarity score generated by the algorithm [number of matches - (0.9)  $\times$  number of mismatched + (1.01 + 0.9)  $\times$  length of gap] is shown. The protein identity ratio is the % identity of aa residues.



*Pst*I fragments of the HC11-1 clone. These cosmid clones were digested with *Bam*HI and subcloned into a pUC18 vector or further digested by *Hinc*II prior to subcloning into pUC18. One of the subclones containing a 7 kb insert, termed pUC 7kb, was subjected to DNA sequence analysis. To this end, the insert of pUC 7 kb was subcloned into M13mp18 and mp19 for DNA sequence analysis as described in the Methods.

The structure of the HC11 gene is shown in Fig. 5(A). The intron-exon organization of the gene was established by comparison of its sequence with that of the cDNA nucleotide sequence. The coding sequence is comprised of three exons separated by two introns of 798 and 381 bp in size, respectively, with consensus splice donor and acceptor sequences at the intron/exon boundaries (29). The first exon contains the 5' untranslated region found in the cDNA, the 23 aa leader sequence plus two amino acids of the predicted mature protein. The second exon encodes 40 amino acids, starting at the second base of the codon for aa 3-Asp and including the second base of the codon for aa 42-Ile. The third exon encodes the remainder of the HC11 protein, aa 43-76, followed by a termination codon and the entire 3' untranslated region (see Figs 5A and 4B). The nucleotide sequence of the coding region of the HC11 gene in pUC 7 kb corresponds precisely with the nucleotide sequence of the four HC11 cDNA clones.

A genomic Southern blot analysis of the HC11 gene was carried out by hybridizing the HC11-1 cDNA probe with DNA derived from the human T cell tumor line REX. As shown in the autoradiograph in Fig. 5(B), a comparatively simple pattern of strongly hybridizing bands was obtained. One band at 9.4 kb in the *Bam*HI digest, one band at 3.8 kb in the *Hind*III digest, and two bands at 2.4 and 1.5 kb in the *Eco*RI digests hybridized with the HC11-1 cDNA probe. We suspect that the weakly hybridizing band at ~5 kb in *Bam*HI and ~5.2 kb in *Hind*III DNA digests results from cross-hybridization of a related gene. These data indicate that the HC11 gene is present in human genomes at either one or a small number of copies per haploid genome.

#### *Expression of HC11 protein in COS cells*

The cDNA sequence of HC11 predicted that it encodes a secretory protein with a processed polypeptide core of ~9 kd. To determine whether or not the HC11 protein could be secreted, the coding region of the full-length cDNA clone, HC11-30, was subcloned into the transient expression vector pmt2 in both orientations and used to transfect COS cells. Subsequently, cells were metabolically labeled with [<sup>35</sup>S]cysteine and supernatants analysed by SDS-PAGE followed by autoradiography. As shown in Fig. 6, the cells that were transfected with plasmid containing HC11 in the sense orientation (+) secreted proteins with apparent MW of 12, 14, 15, and 16.5 kd that were not present in the supernatants of cells transfected with the anti-sense orientation of HC11 (-). This result unequivocally demonstrated that HC11 encodes a secretory protein(s). Whether these different bands represent the protein products of HC11 in differentially glycosylated forms seems likely but remains to be determined.

#### *Comparison of nucleotide and predicted protein sequence of the HC11 gene with members of an inducible cytokine gene family*

The nucleotide sequence of human HC11-30 was compared with sequences in the GenBank nucleotide data base (release gb 57)

using the DASHER algorithm. The only significant homology found in GenBank was to a cDNA clone termed murine JE which was isolated from a cDNA library derived from platelet-derived growth factor (PDGF)-treated BALB/c 3T3 cell fibroblasts (7). Of 592 nucleotides, 429 were identical in comparison of residues 3-601 of HC11 with 20-667 of JE. Likewise, when the predicted protein sequence of human HC11 was tested for homology to the translated GenBank and protein bank (release nbf-18-seq) using the DASHER program, only the translated JE protein showed significant homology to HC11 protein (51.5%). A comparison of the predicted amino acid sequence of HC11, JE, and HC14 is shown in Fig. 7. Note the substantial conservation in primary amino acid sequences among these proteins, including the four characteristic cysteine residues (see below). The sequences diverge after aa residue 68 in the mature protein. Whether these structural similarities and differences are important for function of these proteins is likely but unknown at present.

The spacing and position of the four cysteines in HC11 and HC14 are characteristic of molecules which were identified recently as members of a new inducible cytokine family (4,9). All the sequences in the family are derived from cDNA clones obtained from stimulated cells (monocytes, lymphocytes, or fibroblasts) and are not expressed in the uninduced state (Table 1). Consequently, we compared the nucleotide and protein sequence of HC11 against all the sequences in the cytokine family (Table 2). The protein sequence of HC11 is 27.2-36.3% identical to that of murine TCA-3 (3), human Rantes (4), human LD78 (2), and murine MIP-1 $\alpha$  (5) and MIP-1 $\beta$  (6). Interestingly, the homology among the amino acid sequence of HC11 with HC14 and with JE is much higher, 62.3 and 51.5% identity respectively. A similar pattern of high homology among HC11, HC14, and JE was observed when comparing their nucleotide sequences. This was the case regardless of whether DNA of the coding region or the entire cDNA sequence was compared. Given this overall sequence similarity, it is possible that HC11 or HC14 is the human homolog of murine JE.

Eleven amino acid residues are conserved among the members of this cytokine family (Fig. 7, shaded residues). Nine of the residues are found in the predicted mature protein sequence and are identically spaced in all members of this group. These include four cysteines at aa positions 11, 12, 26, and 52, tyrosine 28, phenylalanine 43, alanine 53, tryptophan 59, and valine 60. This conservation strongly suggested that HC11 and HC14 indeed belong to this new cytokine family. Moreover, since the HC11, HC14, and murine JE are substantially more homologous to one another than to other family members, they appear to define a distinct subgroup. Whether this has implications for the functions of these proteins needs to be determined. One might predict that the functions of HC11 and HC14 are related to one another.

Although we do not know the biological function of HC11 and HC14, several features suggest that the proteins which they encode may be important in some regulatory aspect of hematopoiesis or other cellular systems: (i) their expression is not constitutive (Fig. 2); (ii) they can be induced in macrophages and the monocytic line U-937 by  $\gamma$ -IFN, a known immunomodulator, or by TPA in the premonocytic cell line HL-60 (Fig. 2A and data not shown); and (iii) they are homologous to other

members of a new inducible cytokine family which has been suggested to play a role in inflammation (Table I). Given the ability to express HC11 in COS cells, functional analysis of the recombinant HC11 product can now be pursued

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## Abbreviations

aa	amino acid(s)
DMSO	dimethylsulfoxide
$\gamma$ -IFN	gamma interferon
mAb	monoclonal antibody
MIP	macrophage inflammatory protein
PBL	peripheral blood lymphocytes
SDS - PAGE	sodium dodecylsulfate - polyacrylamide gel electrophoresis
ss-cDNA	single-stranded complementary DNA
TPA	12-O-tetradecanoylphorbol-13-acetate

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## Note added in proof

Since completion of this work, another group has independently identified a cDNA, denoted MCAF (monocyte chemotactic and activating factor), which has an identical predicted amino acid sequence to HC11 (Furutani, Y. et al., *Biochem. Biophys. Res. Commun.* 159:249, 1989).